Chinese Agron. J. 4: 257-268 (1994)
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恆春薯蕷之組織培養
(二)、細胞懸浮培養之建立及Diosgenin含量之測定

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摘要：利用懸浮細胞生長迅速，二次代謝物產量豐富及萃取容易之優點，生產吾人所需生藥成分的技術，目前已受到世界各先進國製藥工業的重視。本研究之目的乃延續恆春薯蕷（*Dioscorea doryophora* Hance）組織培養之研究，嘗試將恆春薯蕷組織培養所產生之癒合組織，建立細胞懸浮培養，並迅速獲致大量的diosgenin，作為合成醫療用類固醇的原料。結果發現，零餘子及節的癒合組織於含0.1mg/l 2,4-D之MS液體培養基中，並以120rpm之速度震盪培養，可建立良好且生長快速的細胞懸浮培養。6%蔗糖較有利於零餘子及節懸浮細胞的生長及乾重形成，但diosgenin的形成卻以3%蔗糖最佳。HPLC的分析發現零餘子來源癒合組織所建立之懸浮細胞，含diosgenin之量高達3.2%，而節部來源癒合組織所建立之懸浮細胞，含diosgenin之量僅及0.3%，顯示不同來源培殖體所誘導之細胞，合成diosgenin之能力各有不同。懸浮細胞合成diosgenin之時間，以懸浮細胞經繼代培養後第10天較高可達3.3%，其餘時間約在2.7～2.9%間。

關鍵語：恆春薯蕷、懸浮培養、二次代謝物、Diosgenin。

Tissue culture of *Dioscorea doryophora* Hance. II. Establishment of suspension culture and the measurement of diosgenin content

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Abstract. **The production of medicinal compounds from cell suspension culture system is one of the promising methods in medicinal industry due to the cells from suspension cultures not only grow faster**

than cells of the intact plants, but also the secondary metabolites produced can be extracted more easily. The purpose of this study was to establish the cell suspension cultures of *Dioscorea doryophora* Hance for the production of diosgenin which can be used as an alternative source for the synthesis of steroids. It was found that a finely dispersed cell suspension culture could be established, when the microtuber- or node-derived callus was cut and cultured on a medium containing MS basal salts supplemented with 0.1 mg/1 2, 4-D, 3% sucrose and shaking at 120 rpm.

Although 6% sucrose was an optimum concentration for the growth and increasing dry weight of the cell suspension culture, 3% sucrose gave better results of the diosgenin production. Analyses by HPLC indicated that both microtuber- and node-derived suspension cells contained diosgenin. As high as 3.2% diosgenin content from microtuber-derived suspension cells was obtained, whereas the node-derived suspension cells contained only 0.3% diosgenin. The diosgenin content varied among cell suspension derived from different source explants. The content of diosgenin in cell suspension was maximum (3.3%) on the 10th day of cultures, and that was 2.7~2.9% for the rest periods of cultures.

Key words: Diosgenin, *Dioscorea doryophora*, Secondary metabolites, Suspension culture.

前 言

利用細胞懸浮培養生產有用的二次代謝物，過去二十年來成功的例子如Chen et al.（1984）及Seitz et al.（1985）報導長春花（*Catharanthus roseus*）利用懸浮細胞生產vinblastine, vincristine及ajmalicine；Fukui et al.（1982）和Morimoto et al.（1986）指出日本黃連（*Coptis japonica*）可利用懸浮細胞生產berberine。Yamamoto and Yamada（1986）提出印度羅芙木（*Rauwolfia serpentina*）可利用懸浮細胞產生reserpine。Fujita et al.（1981a, 1981b）及Tabata（1982）則皆證明紫草（*Lithospermum erythrorhizon*）可利用懸浮細胞生產shikonin及其衍生物。Kaul and Staba（1968），Kaul et al.（1969），Stohs et al.（1974），Tal et al.（1983, 1984a, 1984b, 1984c）及Tarakanova et al.（1979）等皆報導了三角葉薯（*Dioscorea deltoidea*）可利用懸浮細胞生產diosgenin。

台灣特有之恆春薯蕷（*Dioscorea doryophora*）擔根體品質優良，廣爲市場做爲藥用或食用。本科中一部分植物含有diosgenin, diosgenin是一種類固醇皂甘（steroidal saponins），在薯蕷科（Dioscoreaceae）、百合科（Liliaceae）、石蒜科（Amarillidaceae）、玄參科（Scrophulariaceae）及毛地黃屬（Digitalis）植物中，皆存有此成分。以diosgenin爲基本架構，可合成許多醫療用的類固醇（medicinal steroids），如prednisolone、dexamethasone、norethisterone、metenolone等。爲了開發本省自產藥材，提煉主要成分以利製藥之發展及藥用資源之開發，遂對恆春薯蕷進行細胞懸浮培養之研究。

材料與方法

㈠恆春薯蕷細胞懸浮培養之建立

1.材料：

本研究所使用之材料係由恆春薯蕷（*Dioscorea doryophora*）各部位誘導之癒合組織經多次繼代培養而來（Huang et al. 1993）。

2.培養基之製備：

本研究主要以Murashige & Skoog（1962）無機鹽類（MS）爲基本配方，添加各類植物生長調節劑及有機添加物，以1N NaOH或HCl將pH調至5.2±0.1，以121℃,15 lb/in²（1.05 kg／cm²）之壓力殺菌15分鐘。

3.培養環境：

將含有25ml懸浮細胞之125ml三角瓶，置於平面迴轉式震盪器上，於25±1℃之恆溫，以120rpm之速度震盪黑暗培養之。所有懸浮細胞之開始濃度均爲25ml培養基中含有0.5g鮮重之細胞。

㈡Diosgenin含量之測定

Diosgenin在植物體內常與糖基結合，故進行diosgenin含量測定前，可加酸將糖基與diosgenin切斷，而產生free type的diosgenin. Chang（1979）及Lee（1984）均指出以TLC測定diosgenin，展開溶媒爲氯仿：乙酸乙酯＝15：1，呈色劑爲10%H_2SO_4，得到diosgenin Rf值爲0.23，本研究即以上述之方法測定。

以HPLC檢測分析diosgenin，則選用revese phase C_{18} column，檢測波長定於215nm, mobile phase爲methanol：water＝95：5, flow rate爲1.0ml／min並採用7-Ketocholesterol爲internal standard。

本研究所使用之材料爲細胞懸浮培養所產生之細胞。使用之溶媒有甲醇、HPLC級甲醇、HPLC級水、丙酮、氯仿及乙酸乙酯。而使用之試劑則有鹽酸、無水硫酸鈉及氫氧化鉀。矽膠薄層分析係採用E. Merck公司TLC aluminium sheets silica gel 60 F_{254} precoated,

25 sheets 20×20 cm, layer thickness 0.2mm（Art.5554）。

使用之儀器廠牌及型號爲：

High performance liquid chromatography（HPLC）：Shimadzu

Detector：Shimadzu SPD-6A,UV Spectrophotometric detector

Column：Merck,Cat.50838,100 RP-18 Endcapped（5μm）No.011373

5.方法：

(1)Diosgenin之精製：

取diosgenin（Sigma No. D-1634）5g以丙酮再結晶兩次，得白色針狀結晶，以此結晶爲標準品。

(2)Diosgenin之校正曲線：

取精製過之diosgenin精確稱量，以容量瓶分別配製濃度爲2mg/ml, 1mg/ml, 0.5mg/ml等三個濃度，各取10μl與internal standard（7-Ketocholesterol；Sigma No. C-2394濃度：1.0mg/ml）10μl，作HPLC分析測定，重覆二次（圖1）。

(3)材料之萃取及分析測定：

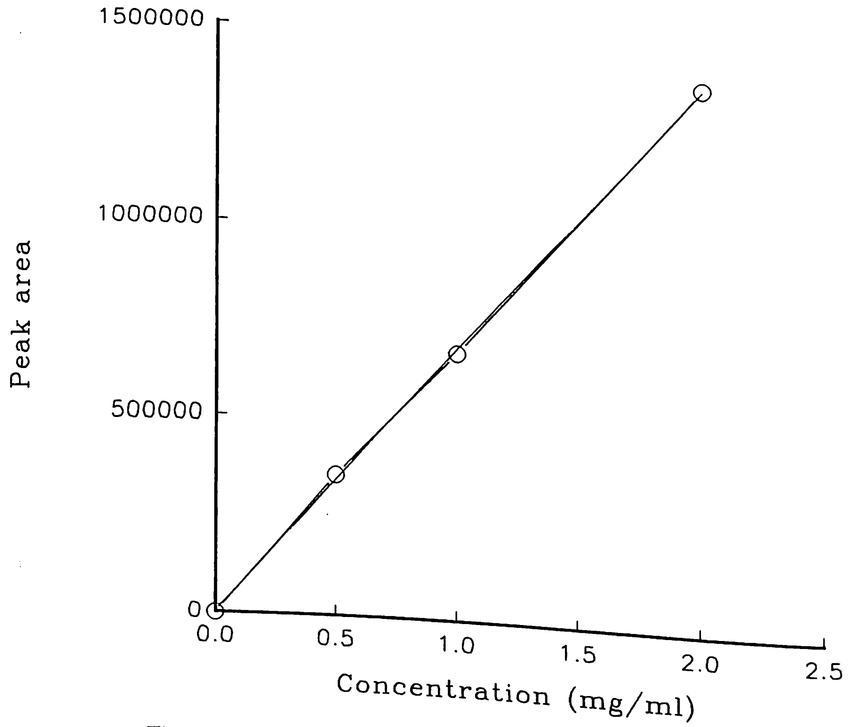

Fig. 1. The regression curve of diosgenin.
Regression Equation:
Y = 642666x − 10667
Coefficient of correlation: 0.9998

取乾燥之前述材料1g，放入soxhlet抽出器中，以甲醇80ml萃取（約10小時；溫度維持於60℃），過濾後，經減壓濃縮；加入5％鹽酸：甲醇＝1：1之混合液150毫升，迴流4小時，反應完後以氫氧化鉀溶液中和至pH8，再以氯仿萃取多次，合併氯仿層，以無水硫酸鈉脫水，再減壓濃縮。將此濃縮液與diosgenin標準品之薄層層析（TLC）相比較。

將上述水解物減壓濃縮乾乾燥後，加入HPLC級甲醇，以微孔過濾器（millipore）過濾，取10μl加10μl internal standard作HPLC分析測定，各作二次。

(三)數據分析方法

本研究之各項數據，則使用ANOVA統計法分析並以Duncan's Multiple Range Test測定處理間之差異。

結　果

(一)植物生長調節劑對恆春薯蕷細胞懸浮培養之影響

將誘導自零餘子（microtuber）及節（node）之癒合組織，經多次繼代培養後，取癒合組織質地鬆脆之部分，以刀片切碎後，各取約0.5g移入含不同auxin濃度之MS液體培養基中，建立細胞懸浮培養（每隔7～10天繼代培養一次），結果如表1及表2所示。零餘子

Table 1. Influence of plant growth regulators on the growth of cell suspension derived from callus of microtuber

MS basic salts with 2,4-D (mg/l)	NAA (mg/l)	Dry weight (20-days old) (g/l)
0.1	0	9.7[a]*
0.2	0	7.6[b]
0.5	0	6.3[d]
1	0	4.8[fg]
2	0	4.6[gh]
4	0	3.1[i]
0	0.1	6.8[c]
0	0.2	6.9[c]
0	0.5	4.9[ef]
0	1	5.1[e]
0	2	4.9[ef]
0	4	4.6[gh]
0	8	3.0[i]

*Means in each vertical column followed by same letter are not significantly different at 5% level using Duncan's multiple range test. Original fresh weight was 0.5g/25ml liquid medium.

Table 2. Influence of plant growth regulators on the growth of cell suspension derived from callus of node

MS basic salts with 2,4-D	NAA	Dry weight (20-days old)
— mg/l —		— g/l —
0.1	0	9.8[a]*
0.2	0	8.5[b]
0.5	0	7.6[c]
1	0	5.1[f]
2	0	5.0[f]
4	0	4.8[f]
0	0.1	7.7[c]
0	0.2	7.6[c]
0	0.5	6.1[d]
0	1	5.3[e]
0	2	4.3[g]
0	4	4.4[g]
0	8	4.1[g]

*Same as Table 1.

及節之懸浮細胞在受測0.1～4mg／l 2,4-D（2,4-dichlorophenoxyacetic acid）的範圍中，以0.1mg/l 2,4-D之MS培養基的生長速率最快：隨著2,4-D濃度增加，零餘子及節的懸浮細胞，生長趨於緩慢。NAA（naphthaleneacetic acid）在0.1～8mg／l的濃度範圍亦顯示相同的趨勢，以0.1mg／l對懸浮細胞的生長最佳，唯其乾重不若0.1mg/l 2,4-D之處理佳。

(二)蔗糖濃度對恆春薯蕷細胞懸浮培養之影響

表3、表4之結果顯示含有6％蔗糖濃度之MS液體培養基，對零餘子及節來源懸浮細胞之乾重增加最多，含4.5％蔗糖次之，而含3％蔗糖濃度之MS液體培養基乾重增加最少。

Table 3. Influence of sucrose concentration on the growth of cell suspension derived from callus of microtuber*

Sucrose concentration — % —	Dry weight (20-days old) — g/l —
3.0	9.5[c]**
4.5	12.3[b]
6.0	16.5[a]

*Basal medium: MS salts supplemented with 0.1 mg/l 2,4-D.
**Same as Table 1.

Table 4. Influence of sucrose concentration on the growth of cell suspension derived from callus of nodes*

Sucrose concentration — % —	Dry weight — g/l —
3.0	9.4^{c**}
4.5	12.5^{b}
6.0	16.3^{a}

*Basal medium: MS salts supplemented with 0.1 mg/l 2,4-D.
**Same as Table 1.

㈢不同來源懸浮細胞diosgenin含量之測定

將零餘子及節來源的懸浮細胞，培養於含有0.1mg/l 2,4-D及3%蔗糖之MS基本鹽類培養基中10天後，取其細胞經乾燥及酸化後，進行HPLC分析測定，結果如表5所示。零餘子來源之懸浮細胞，含3.2% 的diosgenin，而節來源之懸浮細胞，則只含0.3% 的diosgenin。

㈣不同蔗糖濃度培養下之零餘子懸浮細胞diosgenin含量之測定

表6顯示不同蔗糖濃度培養基下生長十天之零餘子懸浮細胞，經乾燥、酸化等步驟處

Table 5. Diosgenin content of cell suspension from node and microtuber*

Source of cell suspension	Diosgenin content — % —
node	0.3^{b**}
microtuber	3.3^{a}

*Basal medium: MS salts supplemented with 0.1 mg/l 2,4-D.
**Same as Table 1.

Table 6. Influence of sucrose concentration on diosgenin content of cell suspension derived from callus of microtuber*

Sucrose concentration — % —	Diosgenin content — % —
3.0	3.1^{b**}
4.5	2.0^{a}
6.0	1.2^{c}

*Basal medium: MS salts supplemented with 0.1 mg/l 2,4-D.
**Same as Table 1.

理後進行HPLC分析，結果如表6所示。於3%蔗糖處理之懸浮細胞，所含diosgenin的量最高達爲3.1%，隨著蔗糖濃度的升高，懸浮細胞產生diosgenin的量遞減，4.5%蔗糖處理爲2%，而6%蔗糖之處理diosgenin僅及1.2%。

(五)不同培養天數懸浮細胞diosgenin含量之測定

將培養於含0.1mg/l 2,4-D及3%蔗糖培養基不同天數之零餘子懸浮細胞，經乾燥、酸化等處理後，作HPLC分析測定，其結果如表7所示。懸浮細胞經10天之培養，所含diosgenin的量最高可達3.3%，之後其含量即稍微下降，在15～20天後仍可維持在2.7～2.9%間。

Table 7. Diosgenin content of suspension cell in different culture duration*

Days after inoculation in suspension culture	Diosgenin content — % —
5	2.7[c]**
10	3.3[a]
15	2.9[b]
20	2.7[c]

*Basal medium: MS salts supplemented with 0.1 mg/l 2,4-D.
**Same as Table 1.

討　論

懸浮細胞因生長快速且均一，所以較癒合組織更適合作生理、生化及成分上的研究。Hunanlt et al.（1988）在懷香（*Foeniculum vulgare* Miller）的細胞培養中指出，建立一個細胞懸浮培養其實非常簡單，因爲使用於固體培養的培養基，同樣也適用於液體培養。Hiraoka（1988）也使用與癒合組織培養相同的培養基（不含agar），建立柴胡細胞懸浮培養。Chang（1991）以與癒合組織生長相同的MS＋1mg/l 2,4-D及0.5mg／l kinetin培養基作爲液體培養基，將台灣白芷的癒合組織切碎後培養，可得一生長良好的細胞懸浮培養。

成功的細胞懸浮培養，決定於起始癒合組織之質地，此外，培養環境亦是重要的影響因素，一般溫度應控制在24～30℃，振盪器的速率約120～160rpm內最適合懸浮細胞之生長。因此，本研究使用之癒合組織其質地良好而脆，來自零餘子及節的癒合組織分生能力較強。同樣地，培養基中的植物生長調節劑亦對懸浮細胞的生長速率有重大的影響。

Berlin et al.（1986）曾提出*Chenopodium rubrum*於含2×10^{-6}M2,4-D液體培養基中可

獲致良好的細胞懸浮液。Misawa（1977）亦指出*Phytolacca americona*於含1mg/l 2,4-D液體培養基中可建立良好細胞懸浮培養。Mehta and Staba（1970）報導*Dioscorea composita*可於含 1mg/l 2,4-D 之液體培養基中建立懸浮培養。Kaul and Staba（1968）,Kaul et al.（1969）及Urmantseva et al.（1984）之研究報告亦指出*Dioscorea deltoidea*在0.1～1 mg/l 2,4-D液體培養基中可獲致良好的懸浮培養，本研究所得結果與上述諸學者之結果極為接近。

Auxins不但在細胞懸浮培養上扮演重要角色，且對二次代謝物的產生舉足輕重。例如*Macuma pruienses*生成L-dopa及*Dioscorea deltoidea*生成diosgenin可被高濃度的2,4-D所誘導（Misawa 1985），但也有報告指出，2,4-D會刺激細胞的去分化且導致二次代謝產物的降低，因此建議以NAA或IAA代替（Tabata & Hiraoka 1976, Tabata et al.1974）。

本研究發現比癒合組織生長所需稍低之2,4-D濃度（0.1mg／l）較有利於懸浮細胞之生長，且2,4-D效果優於NAA，其結果與上述諸學者之論點稍有差異。至於2,4-D或NAA之使用是否會對diosgenin的形成造成影響，則有待進一步之研究。

培養基中添加碳水化合物之主要目的為提供碳源，供給能量及調整滲透壓。一般組織培養的培養基中多使用蔗糖。Yomamoto et al.（1986）報導不同濃度的蔗糖對黃芩癒合組織生長之影響，以3％的蔗糖最好，8％則有明顯之抑制作用；3％蔗糖也最適合Coptis的細胞培養及berberine的生成（Ikuta and Itokawa 1982）。紫草的懸浮培養也以3％蔗糖對細胞及shikonin的生成較好（Fujita et al. 1981b）。

本研究雖顯示在3％～6％蔗糖濃度範圍中，蔗糖濃度愈高對懸浮細胞之乾重增加愈有利，但高濃度的蔗糖卻又對diosgenin之合成有負面的影響，表6顯示3％蔗糖培養基之懸浮細胞所含diosgenin之量最高為3.1％，而6％卻只有1.2％之diosgenin，因此為了diosgenin之含量，懸浮細胞培養基之蔗糖仍以3％為宜。

Suzuki and Matsumoto（1988）發現*Rubia cordifolia*細胞在含有蔗糖、葡萄糖及果糖等不同種類碳源的培養基中，anthraquinone的產量以在葡萄糖的培養基中最多。Ikuta and Itokawa（1982）在Coptis的研究也發現以3％葡萄糖或3％麥芽糖對berberine的生產效果比3％蔗糖好。Chang（1991）在白芷懸浮細胞的研究也認為以3％葡萄糖對imperatorin產量效果較蔗糖好。是否將蔗糖改用葡萄糖，或使用其他種類之碳源，對恆春薯蕷零餘子懸浮細胞產生diosgenin較有利，值得進一步研究及測試。

Chang（1991）報導台灣白芷的癒合組織及細胞懸浮培養，經由TLC及HPLC證明含有imperatorin成分，但含量不高，這可能與當初誘導癒合組織的培殖體有關。台灣白芷的根含imperatorin量約234.75μg／gd.w.，而莖葉所含的量只有11.75μg／gd.w.，因此由葉柄所誘導出來的細胞或癒合組織imperatorin的含量甚低。但Berlin（1988）認為植物細胞皆有全能性，可經由不同分化過程長成一完整植物，也就是由根部衍生之癒合組織所合成之產物，葉或莖的癒合組織一樣可以得到，或許只是量的多寡不同而已，但吾人可以調整培

養條件來改變其成分產量。本研究發現不同培殖體所誘導之癒合組織其diosgenin含量亦各有不同。所建立的懸浮細胞，零餘子來源之癒合組織含量高達3.3%。而由節部來源之癒合組織則只有0.3%。Chen（1985）指出恆春薯蕷(*Dioscorea doryophora*)含diosgenin 3.76%，本試驗檢測發現恆春薯蕷零餘子來源癒合組織所建立懸浮細胞之diosgenin含量爲3.3%，與上述結果極爲相近。由此觀之，未來若考慮以懸浮細胞生產diosgenin，當以零餘子來源癒合組織所建立之懸浮細胞較具實用性。

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收稿日期：83年 5 月12日，接受刊登日期：83年 6 月22日

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